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β-Glucoside Activators of Mung Bean UDP-Glucose: β-Glucan Synthase[1]

II. COMPARISON OF EFFECTS OF AN ENDOGENOUS β-LINKED GLUCOLIPID WITH SYNTHETIC *n*-ALKYL β-D-MONOGLUCOPYRANOSIDES

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ABSTRACT

n-Alkyl (C_6-C_{12}) β-D-monoglucopyranosides have been found to be highly potent activators of mung bean β-glucan synthase *in vitro*, increasing the V_{max} of the enzyme as much as 60-fold and with K_a values as low as 10 micromolar. Activation is highly specific for the β-linked terminal glucose residue; other alkyl glycosides such as, octyl-α-glucoside, dodecyl β-maltoside, 6-lauryl sucrose, 6-lauryl glucose, which lack this structure, are ineffective as activators. Based on the similarities in their structure and effects on β-glucan synthesis under a variety of conditions, it is proposed that the alkyl β-glucosides are structural analogs of the native glucolipid activator of β-glucan synthase isolated from mung bean extracts.

The β-glucan synthase in membrane preparations from a variety of plant sources (5, 7, 11) is markedly stimulated by β-linked glucosides, such as cellobiose, laminaribiose, and gentiobiose. However, these dissacharides do not occur within the cell at the high millimolar concentrations required to activate the β-glucan synthase *in vitro*. In the preceding paper (3), the β-glucan synthase from mung beans was shown to be stimulated *in vitro* by an endogenous, low mol wt material, tentatively characterized as a β-linked glucosyl glycerolipid (Activator-A). In this report, we describe activation of the enzyme by low, micromolar concentrations of alkyl β-monoglucosides and compare their effects to those of the β-linked glucobioses and the native activator. The results permit the formulation of a structure-function relationship for activators of β-glucan synthase, which could have bearing on the mechanism and regulation of β-glucan synthesis *in vivo*.

MATERIALS AND METHODS

Materials. UDP-[^{14}C]glucose was purchased form the Radiochemical Centre, Amersham, U.K. Lauryl 6-sucrose was from Mitsubishi, Japan. Dodecyl β-maltoside was from CalBiochem. Mixed isomers of octyl glucoside was from Pfanstiehl Laboratories Inc., Waukegan, IL. Activator-A was obtained as described in the preceding paper (3). Silica gel-60, TLC plates were from Merck, Germany. TLC plates of polyethyleneimine-cellulose were from Machery-Nagel (Polygram Cel-300 PEI). All other chemicals and enzymes were from Sigma.

β-Glucan Synthase Assay. Washed membranes from mung bean hypocotyl (*Vigna radiata*) were prepared as described in the preceding paper (3). Standard assays were performed at 20°C for 10 min and contained in a final volume of 0.25 ml: 5 μm UDP-[^{14}C]glucose (300 cpm/pmol); 10 mM $MgCl_2$; 2 mM $CaCl_2$; 8% (w/v) PEG-4000; 20 mM EPPS[3]-NaOH buffer (pH 7.5); and washed membranes (5–10 μg protein). Reactions were terminated by addition of 2 ml 24% KOH (w/v) and the 24% KOH insoluble product was determined as described in the preceding paper (3).

Product Digestion. Standard β-glucan synthase reaction mixtures containing 250 μM octyl β-glucoside were terminated, filter washed, incubated with either laminarinase, endo-1,3-β-glucanase, or exocellobiohydrolase, and percent digestion and digestion products analyzed as described in the preceding paper (3).

Chromatographic Analysis. For determination of UDP-Glc substrate depletion, standard β-glucan synthase assay mixtures were deproteinized by heating at 100°C for 3 min and centrifugation. Aliquots (20 μl) were applied to polyethyleneimine-cellulose plates and resolved in 0.2 M KH_2PO_4 (pH 4.0). The ^{14}C-labeled UDP-Glc spot was detected by autoradiography, excised, and counted. Percent degradation was determined by comparison to reaction mixtures which were boiled for 3 min prior to addition of UDP-Glc.

Sugar and glycerol content was analyzed by descending paper chromatography on Whatman No. 4 for 20 h using *n*-propanol:ethyl acetate:water (7:1:2) as solvent and sulphuric acid charring for visualization.

Enzymic and Chemical Treatments. Invertase (yeast, 1 unit/ml) and α-glucosidase (yeast type I, 2 units/ml) digestions were performed by incubating 0.25 μmol of alkyl glycoside with enzyme in either 0.25 ml NaH_2PO_4 (pH 5.0), or EPPS buffer (pH 7.0), respectively, at 30°C for 30 min. Periodate oxidation was carried out in a 1 ml solution of 100 mM sodium metaperiodate and 10 mM octyl β-glucoside at 4°C for 16 h in the dark. The reaction was quenched by addition of 0.1 ml ethylene glycol, 100 mg sodium borohydride was added and after 1 h at 20°C, the mixture was neutralized by addition of acetic acid. The product was applied to a 0.5 ml DEAE-cellulose column and eluted with water.

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[3] Abbreviations: EPPS, *N*-(2-hydroxyethyl-)piperazine-*N*′-3-propane sulfonic acid; CHAPS, 3-([3-cholamidopropyl]-dimethylammonio)-1-propanesulfonate.

RESULTS

Effects of Alkyl Glycosides on β-Glucan Synthase. The β-glucan synthase in washed membranes is stimulated up to 60-fold in the presence of micromolar concentrations of *n*-alkyl β-D-monoglucopyranosides (alkyl glucosides). This activation effect is comparable to that produced by the native activator (Activator-A) and, as exemplified in Figure 1 for octyl-β-glucoside, displays hyperbolic saturation kinetics with respect to glucoside concentration. Within the homologous series tested, the potency of alkyl β-glucosides—as reflected in decreasing K_a values—rises significantly with increasing alkyl chain length (Table I). In contrast, at saturating concentrations of the various alkyl β-glucosides, the maximal fold stimulation attained varies only slightly with alkyl chain length. An interesting exception is the effect of octyl β-D thioglucopyranoside, which is three times more potent than the corresponding glucoside derivative and moreover, among all the activators tested, exerts the highest overall stimulatory effect. Methyl β-glucoside proved to be only marginally stimulatory (two- to threefold) and did not show a saturation effect in the concentration range of up to 5 mM.

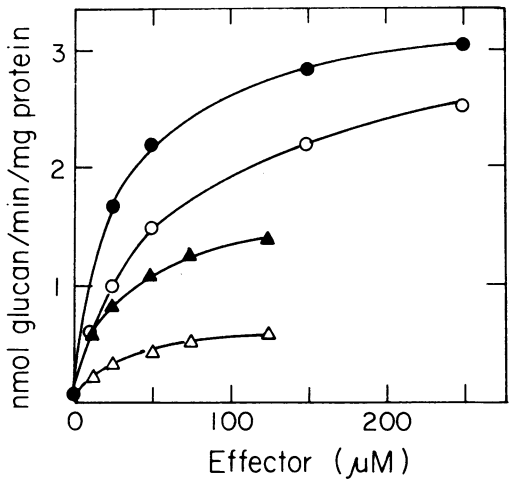

FIG. 1. Effect of octyl β-glucoside and Activator-A on β-glucan synthase activity. β-Glucan synthase was assayed in standard reaction mixtures (8 μg protein washed membranes) supplemented at the concentrations indicated with either activator-A (▲) or octyl β-glucoside (○), in the presence (shaded symbols) or absence (open symbols) of 8% PEG-4000.

Table I. *Potency of β-Glucan Synthase Activators*

β-Glucan synthase was assayed under standard conditions (8 μg protein washed membranes), in the presence of varying concentrations (1 μM–1 mM) of the various β-glucoside activators, and in the absence and presence of 8% PEG-4000. K_a is the concentration required for half-maximal stimulation of the enzyme. Fold-stimulation refers to the ratio of activity at saturating concentration of activator to basal activity.

Activator	K_a		Stimulation	
	−PEG	+PEG	−PEG	+PEG
	μM		*-fold*	
Activator—A	25	25	9	18
Hexyl glucoside	175	120	40	45
Heptyl glucoside	100	70	45	50
Octyl glucoside	50	25	45	50
Octyl thioglucoside	20	10	50	70
Decyl glucoside	15	10	50	50
Cellobiose	200	120	20	25
Laminaribiose	250	120	20	25

While glucose, at concentrations up to 10 mM, has no effect on β-glucan synthase activity, the two β-linked diglucosides, cellobiose and laminaribiose, as previously reported (2), markedly stimulate enzyme activity, but with significantly higher K_a values than those of the alkyl glucoside series. Other glucose-containing dissacharides tested, sucrose, maltose, and melibiose, had no effect at concentrations up to 10 mM.

Among the alkyl glycosides and related compounds which did not stimulate synthase activity when tested in the 10 to 5000 μM range are: methyl-α-glucoside, 6-lauryl sucrose, dodecyl β-maltoside, 3-*O*-methyl-α-glucoside, glucocerebroside (up to 2 mM), stearoyl dihydroglucerebroside (up to 2.5 mM), dolichol monophosphate (up to 1 mM), and mono- and digalactosyl diglycerides (up to 1.5 mM). A mixed isomeric preparation of octyl α- and β-glucoside only slightly stimulated synthase activity (two- to threefold at 1 mM). Furthermore, 6-lauryl sucrose could not be made effective as an activator following treatment with invertase, which yielded fructose, as judged by TLC analysis, and presumably 6-lauryl glucoside as products. On the other hand, treatment of the inactive dodecyl β-maltoside with α-glucosides rendered it fully effective as an activator. In order to ascertain that an intact C-1 linked glucosyl residue is a necessary structural requirement for activation, octyl β-glucoside was treated with sodium metaperiodate followed by sodium borohydride reduction. The product of this treatment, presumably the glycosidic octyl-C_5 polyolic derivative, as judged by release of glycerol following acid hydrolysis, was found to be ineffective in either stimulating or in interfering with synthase stimulation by untreated octyl β-glucoside. Similarly, octanol (up to 200 μM) was ineffective in these capacities.

The following detergents, in absence of any β-glucoside activator in the assay, at concentrations from 10 μM to 10 mM had no stimulatory effect on β-glucan synthase activity: digitonin, CHAPS, Lubrol P-X, Lubrol 12A9, Brij 58, Tween 20, and Tween 80. Two detergents, SDS and Triton X-100, at all concentrations tested, were inhibitory.

The activation of β-glucan synthase activity produced in the presence of the alkyl β-glucosides is apparently reversible; membranes exposed to 250 μM octyl glucoside lose all of their enhanced synthase activity upon washing, which can subsequently be restored upon readdition of the glucoside. The stimulatory effect cannot be attributed to inhibition of possible substrate depletory reactions (6) since more than 90% of the initial UDP-glucose added could be recovered from unsupplemented reaction mixtures, as judged by chromatographic analysis (data not shown).

Modulation of Reaction Kinetics by Native and Exogenous Activators. In the absence of added activating factors, the membranous β-glucan synthase reaction displays sigmoidal kinetics with respect to UDP-glucose concentration. In the presence of either activator-A, alkyl glucoside, or cellobiose, the UDP-Glc-dependence of the enzyme is shifted to a hyperbolic curve; stimulated enzyme activity results from an increase in both V_{max} and the apparent affinity for the substrate (Fig. 2).

Stimulation of the enzyme by the various activators is potentiated in the presence of 8% PEG-4000 (Fig. 1; Table I). While this effect is reflected in reduced K_a values for all exogenous activators, the effect of PEG is to increase the over all fold stimulation achieved by native activator. Significantly, the maximal fold stimulation produced by the alkyl glucosides or cellobiose was unaffected in the presence of saturating amounts of native activator.

Analysis of the Reaction Product. The ^{14}C-labeled 24% KOH insoluble glucan product formed from UDP-[^{14}C]glucose in the presence of octyl glucoside was analyzed for the nature of the β-glycosidic bond configuration, by exocellobiohydrolase and

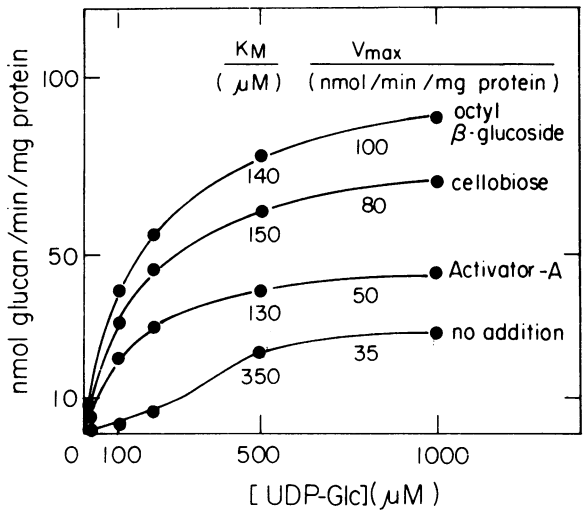

FIG. 2. Effect of variations of UDP-glucose concentration in the presence of various activators of β-glucan synthase. β-Glucan synthase was assayed under standard conditions (8 μg protein washed membranes) at varying concentrations of UDP-Glc and, as indicated, activator-A (50 μM), octyl β-glucoside (200 μM), and cellobiose (500 μM). K_m and V_{max} values were calculated from Lineweaver-Burke plots. $S_{0.5}$ was estimated from the activity at the maximal substrate concentration tested.

laminarinase digestion, followed by chromatographic analysis of the products formed. As with the native activator stimulated reaction (3) the glucan product was found to consist of both β 1-3 and β 1-4 glucosyl linkages in variable proportions. Some of the product samples were nearly completely digested by laminarinase, to yield laminaribiose and glucose as the major products. Other samples were almost totally digested by exocellobiohydrolase to form cellobiose and glucose. No correlations have yet been found between the isomeric nature of the β-glucan polymer product and either experimental conditions or enzyme preparations.

DISCUSSION

In the preceding paper (3) the supernatant fraction derived from mung bean extracts was shown to contain a low mol wt factor, characterized as a β-linked glycolipid, which is highly potent in stimulating membranous β-glucan synthase activity. Similar activation can be achieved using synthetic alkyl β-monoglucosides, ranging from the hexyl to the dodecyl derivatives. The effect does not appear to be the result of detergent action in solubilization of the enzyme, since activation is observed at concentrations of alkyl glucoside well below those required in their capacity as solubilizing agents (8). Moreover, of the various alkyl glycosides tested, only alkyl β-monoglucosides stimulated the enzyme, indicating that activation is related to specific structural features of the alkylated sugar moiety, rather than just to the overall amphipathic nature of the compound.

Based on the materials examined, the β-linked terminal glucose residue, common to the stimulatory alkyl (hexyl-dodecyl) β-glucoside series, appears to fulfill an essential structural requirement for synthase activation. Thus, other alkyl glycosides, in which terminal glucose is in α-linkage (dodecyl β-maltoside), glucose is not in terminal position (lauryl 6-sucrose), glucose is not C-1 linked (lauryl 6-glucose), or terminal glucose has been extensively modified (periodate/borohydride-treated octyl β-glucoside), do not activate the enzyme. The stereospecificity in the structural requirement for enzyme activators is also reflected in the low stimulatory activity of an α- and β-linked octyl glucoside isomer mixture, suggesting that the α-anomer might actually be inhibitory. Presumably, although not yet available, alkyl β-cellobiosyl derivatives should be potent activators of the β-glucan synthase.

Alkyl chain length is also a determinant in the active structure of the alkyl β-glucosides. Within the homologous series tested, the apparent affinity increases in proportion to increasing hydrocarbon chain length, while the maximal fold stimulation achieved is independent of this factor. Thus, the K_a for activation by dodecyl β-glucoside is six times lower than that for the hexyl derivative, but at saturating concentrations both compounds exert similar stimulatory effects. Based on these data, and the low potency of methyl β-glucoside, the hexyl group appears to fulfill a minimum requirement for activator structure. Since the lipophilicity of these compounds is proportional to their alkyl chain length, this effect might be explicable in terms of increased access to the hydrophobic environment of the membrane-bound synthase. However, additional structural features also appear to play a role in the interaction of activator with the membranous synthase system, as indicated by the high potency of octyl β-thioglucoside relative to that of even the most lipophilic alkyl glucoside tested. The fact that digitonin, which contains terminal glucose in β-linkage, cannot substitute for the activating factor suggests that the presence of nonglucose sugar residues and/or of a rigid hydrophobic moiety may be detrimental to this interaction.

Digitonin, at low concentrations, has frequently been reported to stimulate membrane-bound glucan synthase activity (1, 5, 6); the effect appears to be due to the ability of digitonin to facilitate access of substrate to right-side-out vesicles and is independent of any effects on the interaction of β-glucoside with the enzyme (5). Our observations that digitonin does not affect the enzyme may be due to the nature of the sideness of vesicles produced during cell rupture in the presence of PEG. Kauss and Jeblick (10) and Wasserman and McCarthy (12) have also described the effects of various amphipathic molecules on β-glucan synthase activity, but the relationship of these studies to ours is unclear, since those studies were carried out in the presence of saturating cellobiose.

Maximal stimulation of the enzyme by alkyl glucosides is not affected by the native, exogenously added activator, suggesting that both the synthetic and natural materials affect the same binding site and hence, a common rate-limiting step in β-glucan synthesis. Considering this, together with their common structural features, it appears that the alkyl glucosides act as structural analogs of the native β-linked glucolipid activator. A similar relationship might exist between the native activator and the β-linked glucosyl dissacharides, cellobiose, laminaribiose, and gentiobiose, the former sugar being traditionally employed at millimolar concentrations to promote enzyme activity (5, 7, 11). Given that the native activator contains more than one β-linked glucosyl residue, these dissacharides might sufficiently resemble such a structure to activate the enzyme, although with a considerably lower affinity. As with alkyl β-glucosides, β-diglucoside activation shows a rigid stereospecific requirement for the β-linkage, exemplified in the inability of maltose, at comparable concentrations, to stimulate enzyme activity.

All of the activators modulate the kinetics of the reaction in the same fashion, shifting the rate dependence on UDP-Glc concentration from sigmoidal to hyperbolic kinetics. Similarly, the efficacy of all the activators, manifested in either reduced K_a or increased V_{max} values, is enhanced in the presence of polyethylene glycol, which alone does not affect basal activity. These effects on reaction kinetics by native activator and alkyl glucoside described here for membrane-bound enzyme have also been observed in enzyme solubilized from membranes by 1% digitonin (M Benziman, T Callaghan, unpublished results). The similar patterns of synthase activation produced by the various activators lends further support to the possibility that all these effectors share a common mechanism of action.

The marked activation of β-glucan synthases by exogenously added β-linked diglucosides indicated to many workers that these compounds, albeit at high concentration requirements, mimick an analogous, endogenous factor acting *in vivo*, but which is presumably lost in the course of enzyme preparation. The β-linked glucolipid activator(s) from mung bean described here and in the preceding paper are good candidates for the postulated factor. The findings that alkyl β-glucosides are also highly potent in stimulating the enzyme permit the formulation of a structure-function relationship for activators of β-glucan synthase. The striking requirement for the β-linked terminal glucosylated hydrophobic moiety suggests several possible modes of interaction for the native glucolipid activator with the membrane-bound glucosyl transferase. One compelling possibility is that the interaction is covalent, involving the glucosylation of the terminal hexose of the activator, acting as either a primer for *de novo* glucan chain synthesis or as a carrier group participating in the transfer of glucose residues to the elongating β-glucan chain. Alternatively, the glucolipid might interact with the enzyme as a positive allosteric effector.

Further study of the glucolipid-synthase interaction might be of broad significance, when considering the recently postulated interrelated mechanism for callose and cellulose synthesis, in which both β 1-3 and β 1-4 linked polyglucans might be formed under altering conditions by the same enzyme complex (4, 9). In this light, the stimulation of both β 1-4 and β 1-3 bond formation by alkyl glucoside is encouraging, in being compatible with a mutual enzymic step in the synthesis of both isopolymers. Notwithstanding the precise mechanism of glucolipid activation, the discovery of chemically defined, high affinity activators of β-glucan synthase should be of practical value in current schemes to purify and characterize the catalytic and possible regulatory subunits of the enzyme.

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